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Antioxidant Properties of Pecan Shell Bioactive Components of Different Cultivars and Extraction Methods

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ANTIOXIDANT PROPERTIES OF PECAN SHELL BIOACTIVE COMPONENTS OF DIFFERENT CULTIVARS AND EXTRACTION METHODS

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ABSTRACT

Pecan nuts are a highly valued but underutilized crop. Pecan production generates nearly 150 million pounds of shell by-product annually in the United States, of which approximately 6 million pounds are attributed to Louisiana. Pecan shells are a rich source of various phenolic compounds with potential antioxidant properties. The main objective of this study was to determine the effect of pecan variety and method of extraction on the phenolic content and antioxidant activity of pecan shell extracts. A total of 20 different pecan cultivars from the same orchard, under similar growing conditions were processed to obtain defatted shell powder of about 50-100 μm size. The defatted shell powders (hexane 1:20 W/V) were then subjected to distilled water (at 98˚C for 30 min) and ethanol solid-liquid extraction (at 160 rpm for 1 h) processes, respectively. The resultant crude aqueous and ethanol extracts were lyophilized, and the obtained powdered extracts were analyzed for total phenolics and antioxidant activity by Folin-Ciocalteu, and DPPH· free radical assays, respectively. Crude and acid hydrolyzed (acidified methanol 1% HCl V/V, 2 h, 22^{o}C) extracts from Nacono and Caddo cultivars were analyzed by reverse phase HPLC. Acidified methanol soluble components of Nacono ethanolic extracts where further characterized by flow injection electrospray ionization mass spectrometry (FIA-ESI-MS). Pecan cultivar significantly affected (P<0.05) the phenolic content and antioxidant activity of shell extracts. Total phenolic content of shell ethanolic extracts ranged from 304.2 (*Caddo*) to 153.54 (*Cherokee*) mg GAEg^{-1} dry extract with an average of 210.02±7.3 mg GAEg^{-1} and were significantly greater (P<0.05) than those obtained by aqueous extraction, which ranged from 253.75 (*Curtis*) to 114.63 (*Jackson*) with an average of 168.38±6.8 mg GAEg^{-1} of dry extract. Antioxidant activity of ethanolic extracts ranged from 840.6 (*Maramec*) to 526.74 (*Caper Fear*) and averaged 659.70±21 mg TEg^{-1},

while aqueous extracts ranged from 934.9 (*Curtis*) to 468.3 (*Elliot*) with an average of 619.42±22 mg TEg^{-1}. Acid hydrolysis removed interfering components from crude extracts and allowed for the elucidation of two peaks by RP-HPLC . The most abundant peak was attributed to gallic acid derivatives, and the other did not correspond to phenolic standards used for comparison. The major components identified by FIA-ESI-MS in acid hydrolyzed Nacono shell extracts were lignin degradation products lignols, dilignols, trilignols, and oligolignols. Monolignol fragments of G-unit isobaric dilignol were widespread. The findings of this study show promise to enhance Louisiana pecan revenue streams by utilizing pecan shells as an alternative natural source of antioxidants for use in various food applications.

1. INTRODUCTION

Pecan are one of the only native plants commercially cultivated in the United States. Nearly 300 million pounds of pecans valued at over 500 million dollars are produced annually, in the U.S. (NASS, 2018). The edible seed, or kernel of the pecan is highly desired for its nutritive and sensory properties. High consumption has been associated with reduced risk for cardiovascular disease, Alzheimer's, Parkinson's, and lower oxidative stress in cells.

The shell protective layer constitutes nearly 50% of the mass of a pecan nut. Currently, uses for nut shell are limited, but include particle board fill, lost circulation material following oil drilling, and mulch for gardening (Worley, 1994). In many cases, the shell is a waste problem. Potential novel applications for pecan shells has emerged with rising demands for natural, or non-synthetic food products. Previous works have shown that pecan shell is a rich source of phenolic compounds. These compounds have antimicrobial and antioxidant properties that can be exploited for use in natural food products.

Many factors such as growing region, cultivar, cultivation method, and harvest year have been shown to significantly affect the bioactive content and antioxidant activity of pecan extracts (Rosa, Alvarez-Parrilla, & Shahidi, 2011; Rosa et al., 2014; Villareal-Lazoya, Lombardini, & Cisneros-Zevallos, 2007; Prado et al., 2009, 2013, & 2014; Malik, Perez, Lombardini, Cornacchia, Cisneros-Zevallos, and Bradford, 2009). Louisiana pecan production generates nearly 6 million pounds of shell by-product annually. The antioxidant activity of shell bioactive compounds from pecan cultivars grown in Louisiana has not been studied. There is a lack of comparative studies on the efficacy of extraction methods to yield the most potent extracts.

The main objectives of this study are: (i) To evaluate the effect of cultivar on the phenolic content and antioxidant activity of shell extracts of pecans grown in Louisiana, United States, (ii) To determine the effect of extraction method on the phenolic content and antioxidant activity of pecan shell bioactive components, and (iii) To characterize the bioactive components in pecan shell extracts.

1.1. References

1. Malik, N., Perez, Lombardini, J. L., Cornacchia, Cisneros-Zevallos, R., & Braford, J.(2009). Phenolic compounds and fatty acid composition of organic and conventional grown pecan kernels. *J Sci Food Agric, 89*, 2207-2213. doi:10.1002/jsfa.3708

2. Prado, A. C. P., Aragão, A. M., Fett, R., & Block, J.M. (2009). Antioxidant Properties of Pecan Nut [Carya illinoinensis (Wangenh.) C. Koch] Shell Infusion. *Grasas y Aceites*, *60* (4), 330-335. doi: 10.3989/gya.107708

3. Prado, A. C. P., Manion, B. A., Seetharaman, K., Deschamps, F. C., Arellano, D. B., & Block, J. M. (2013). Relationship between antioxidant properties and chemical composition of the oil and the shell of pecan nuts [*Caryaillinoinensis* (Wangenh) C. Koch]. *Industrial Crops and Products*, *45*, 64-73. doi: 10.1016/j.indcrop.2012.11.042.

4. Prado, A. C. P., Silva, H.S., Silveira, S.M., Barreto, P. L. M., Vieira C. R. W., Maraschini, M., Ferreira, A. R. S., & Block J. M. (2014). "Effect of the extraction process on the phenolic compounds profile and the antioxidant and antimicrobial activity of extracts of pecan nut [Carya illinoinensis (Wangenh) C. Koch] shell." *Industrial Crops and Products, 52*, 552-561. doi: 10.1016/j.indcrop.2013.11.031

5. Rosa, L. A., Alvarez-Parrilla, E., & Shahidi, F. (2011). Phenolic Compounds and Antioxidant Activity of Kernels and Shells of Mexican Pecan (*Carya illinoinensis*). *J. Agric. Food Chem.*, *59*, 152-162. doi: 10.1021/jf1034306

6. Rosa, L. A., Vazquez-Flores, A. A., Alvarez-Parrilla, E., Rodrigo-Garcia, J., Medina-Camposc, O. N., A´ vila-Nava, A., Gonza´ lez-Reyes, S., & Pedraza-Chaverri, J. (2014). Content of major classes of polyphenolic compounds, antioxidant, antiproliferative, and cell protective activity of pecan crude extracts and their fractions. *Journal of Functional Foods*, *7*, 219-228. doi: 10.1016/j.jff.2014.02.008

7. United States Department of Agriculture National Agriculture Statistics Service (NASS). (2018). National Statistics for Pecan. Retrieved from https://www.nass.usda.gov/Statistics_by_Subject/result.php?7345CB67-26CC-3917-AF5BC7828216D668&sector=CROPS&group=FRUIT%20%26%20TREE%20NUTS&o

mm=PECANS

8. Villarreal-Lozoya, J. E., Lombardini, L., & Cisneros-Zevallos, L. (2007). Phytochemical constituents and antioxidant capacity of different pecan [*Carya illinoinensis* (Wangenh.) K. Koch] cultivars. *Food Chem., 102*, 1241-1249. doi: 10.1016/j.foodchem.2006.07.024

9. Worley, R.E. (1994). Pecan Physiology and Composition. In: Santerre C.R. (eds) *Pecan Technology* (pp. 39-48). Springer, Dordrecht.

2. LITERATURE REVIEW

2.1. General introduction

2.1.1. Trends in retail food

Consumer consciousness to potential health risks associated with synthetic chemical usage in the production or manufacturing of food products is a major market driving force in the United States. In a survey conducted by Nielsen (2015) 29% of U.S. respondents ranked “all natural” and “no artificial colors” as very important in food purchasing decisions. Other popular consumer food trends include organic, minimally processed, and fresh. Value added products that provide health promoting benefits are also gaining popularity. In response, food companies have flooded the retail market with new products. Now 3 out of 4 conventional grocery stores and nearly 20,000 natural food stores sell organic products (ERS, 2017). Increasingly, people are trying products from this sector. Bioactive compounds are being studied as potential natural sources of functional ingredients for various food applications.

2.1.2. Definition of plant bioactive

Plant bioactive compounds are secondary metabolites that can elicit a toxicological or pharmacological effect in animals and other organisms (Bernhoft, 2008). These compounds are produced to help a species cope with its specific environmental stresses and maintain overall health (Davidson, Critzer, & Taylor, 2013). Plant life thrives in diverse ecosystems, which suggests that an enormous number of bioactive compounds with different chemical structures and functionalities must exist in nature. Blomhoff (2010) surveys bioactive components found in many plants.

2.1.3. Phenolic compounds

Phenolics represent a major group of bioactive compounds found in plants. Their simplest chemical form is a hydroxyl group attached to a benzene ring. Phenylpropanoids comprised of a 6-carbon phenyl group and a 3-carbon side chain are the backbone of phenolic biosynthesis in plants (Ayabe, Uchiyama, Aoki, & Akashi, 2010). They are synthesized from phenylalanine and tyrosine amino acids precursors. Phenylalanine ammonia lyase catalyzes the conversion of phenylalanine to cinnamic acid, which is converted to *p*-coumaric acid by cinnamate 4-hydroxylase, and finally 4-coumaroyl CoA ligase catalyzes the production of *p*-coumaroyl CoA. Downstream modifications to the 4-coumaroyl-CoA through various enzyme catalyzed pathways results in the production of monolignols, flavonoids, phenolic acids, coumarins, and stilbenes (Deng & Lu, 2017).

Phenolic compounds are recognized as therapeutic antioxidative agents that reduce free radical induced cellular stress (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005; Vladimir-Knežević, Blažeković, Štefan, & Babac, 2012) and modulate the gut microbiome (Selma, Espín, & Tomás-Barberán, 2009). Phenolic compounds have antibacterial efficacy towards spoilage microorganisms and foodborne pathogens. Papuc, Goran, Predescu, Nicorescu, and Stefan (2017) gives an extensive review on the structures, classifications, sources, and applications of polyphenolics involved in prolonging the shelf life of meat products. Pecan shell phenolic extracts have been shown to have *in Vivo* antimicrobial properties to *Listeria monocytogenes*, artificially inoculated on the surface of poultry (Caxambu et al., 2016).

2.1.4. Extraction of plant bioactive compounds

Bioactive compounds produced by plants typically must be extracted from their natural matrix prior to use. Extraction is the isolation of a component from its existing matrix. The common goals of all extractions are to separate an analyte from its matrix, remove interferences, concentrate analyte to one phase, and provide analytical reproducibility independent of sample matrix variation (Smith, 2003). The selection of an extraction procedure is dependent on several factors such as the nature of the sample, characteristics of the target compounds, feasibility, and the overall purpose for extraction. Azmir et al. (2013) discusses different methods used to extract bioactive compounds from plants. Traditional methods are based on the extraction power of water or organic solvents, along with agitation, and heat to penetrate samples and bind to the analyte. The typical procedure to extract plant bioactive components involves: 1. Sample processing to dry and reduce particle size with grinding to increase surface area. 2. Treatment with various solvents which bind the analyte of choice 3. Centrifugation or filtration to remove insoluble materials. 4. Concentrating the extracts by evaporating or lyophilizing the solvent. This step is required for isolated components to be later use in downstream applications.

The efficacy of traditional solvent extraction methods is dependent on several variables including the presence of interfering components, sample characteristics (i.e. particle size, stability, and chemical make-up), and the extraction parameters (i.e. time, temperature, pressure, agitation, and solvent choice)(Azwanida, 2015). The most critical factor to consider is the solvent polarity, which is generally described by the polarity index or dielectric constant (Snyder, 1974). A solvent will bind and dissolve compounds of similar polarity. Azmir et al. (2013) has provided a list of common solvents and plant bioactive

compounds that they extract. Advantages of traditional extraction methods include ease of operation and low processing cost. Disadvantages include, low selectivity, prolonged extraction time, reduced extract consistency, the need for a solvent removal step, and depending on the time and temperature requirements thermal degradation to target molecules can occur (Joana Gil-Chávez et al., 2013). The most common traditional extraction methods include maceration, infusion, percolation, Soxhlet, decoction and hydro distillation. Low selectivity means that separatory steps following extraction are often necessary before characterization or utilization. Smith (2003) provides an in-depth review of sample preparatory techniques. These basic methods have been improved for different samples. This is beyond the scope of this paper however, in-depth reviews have been published on this topic (Azmir et al., 2013; Sasidharan, Chen, Saravanan, Sundram, & Yoga, 2011).

2.1.5. Challenges with application of preservatives in food products

There are significant technical challenges involved with the application of any new food preservative. The compounds first must be isolated or concentrated and characterized. A delivery system must be developed. Then, the treatment dosage must be optimized. In any case, the food matrix under evaluation may react with the preservative and produce undesirable changes to appearance or flavor characteristics. The nature of a food, such as its pH, storage conditions or hydrophobicity may decrease the effectiveness of the preservative. Potential adverse reactions are a major concern for any new concentrated bioactive component. Therefore, they must be cautiously evaluated for toxicity, prior to application on any product intended for use in humans. Lucera, Costa, Conte, & Nobile (2012) gives an in-depth review on the application of natural compounds to food.

2.2. Pecans

2.2.1. General description and cultivation

Pecan [*Carya illinoinensis* (Wangenh) K. Koch] a species of hickory tree native to North America, is commercially cultivated in 14 states for its edible seed. In 2017, more than 270 million pounds of pecan nuts were cultivated in the United States, valued at over 500 million dollars. The major production states (Georgia, New Mexico, and Texas) account for approximately 75% of the total production (NASS, 2018).

Pecan nut development begins with pollination by the shed of catkins to female flowers that are abundant on the tree. Upon pollination, nut maturation occurs over two distinct phases. Phase one (May) occurs between the time of pollination to the shell hardening (August). In this time the nut goes through rapid growth in size, however the embryo or future edible kernel is in a state of slow growth. The second phase begins in august as the embryo reaches full size. At this point, the shell thickens, and kernel filling starts. Kernel filling involves the incorporation of nutrients (lipids, protein, acid-hydrolysable carbohydrates, and minerals) from outside the fruit (Worley, 1994). Around November, full maturation has occurred and the fleshy hull splits. The developed nut is dropped from the tree and awaits harvesting. Following harvesting the pecan nuts are typically treated with a conditioning step with either chlorinated or boiling water. Post-harvest treatment steps loosen the shell layer from edible seed or kernel (NMSU, 2005).

Pecans are categorized as either native or improved varieties (cultivars). Native or “wild” pecans typically have thick shells and a low kernel percentage or shell out weight of around 30%. (Worley, 1994). These properties make them less desirable for commercial cultivation. Improved cultivars have been developed through selectively breeding for characteristics

more favorable for production (i.e. disease resistance) by grafting or budding. They dominate the U.S. pecan market, accounting for approximately 93% of the total crop (NASS, 2018). Depending on the specific cultivar, around 50% of the harvested pecan mass is shell weight (Worley, 1994). Current uses for pecan shells are limited and they are worth little value to shellers.

2.2.2. Reported bioactive properties of pecan shells

Previous studies have shown that pecan shells are a rich source of phenolic compounds with potential antioxidant and antimicrobial properties that could be used to enhance the shelf-stability and safety of other food products (Villareal-Lozoya, Lombardini, & Cisneros-Zevallos, 2007; Prado, Aragão, Fett, & Block, 2009; Caxambu et al., 2016) . Many factors have been shown to significantly affect the phenolic content and antioxidant activity of pecan isolates. The effect of pecan cultivar has been studied. Prado, Aragão, Fett, & Block (2009) used distilled water (98^{o}C) to extract 3 different batches of pecans consisting of approximately 50% Barton, Shashone, Shawnee, Choctaw, and Cape Fear. The average phenolic content was 138±26 mg GAEg^{-1} and antioxidant activity 572±102 mg TEACg^{-1} (Folin-Ciocalteu and DPPH) was significantly affected by cultivar. In another study by Prado and other (2013), year of harvest significantly affected total phenolics, and dry matter yield of pecan extracts obtained using distilled water at 98^{o}C followed by spray drying. Principal component analysis showed a positive correlation between total phenolics, antioxidant activity (ABTS and DPPH), fiber, protein, and color of powdered pecan shell. Increased shell antioxidant activity was associated with kernel cultivars with highest oil content.

Another work by Prado et al. (2014) studied the effect of extraction method on the phenolic content and antioxidant activity of Barton pecans harvest in Brazil, 2011. Total

phenolic content and antioxidant activity colorimetric assays were performed on crude extracts. Extraction method did not significantly affect the extraction yield when comparing distilled water (98^{o}C, 10 min) and ethanol (160 rpm for extractions). However, extraction yield for either was significantly greater than that of supercritical CO_2 with 10% ethanol as a co-solvent. The authors concluded that extracts obtained through distilled water extraction followed by spray drying were significantly greater in total phenolic content (TPC 590.78±4.41 mg GAEg^{-1}) and antioxidant capacity (DPPH 1210.97±25.24 mg TEg^{-1}, ABTS) compared to the other extraction methods evaluated. Supercritical CO_2 extracts contained significantly fewer phenolics and condensed tannins, and very little antioxidant activity.

Villareal-Lozoya, Lombardini, & Cisneros-Zevallos, 2007 measured the phenolic content, condensed tannin content, and antioxidant activity (Folin-Ciocalteu, Vanillin assay, and DPPH) of acetone: water (70:30 v/v) extracts from defatted shells and kernels of six cultivars. All cultivars, with exception of Kiowa, were grown at the USDA Experiment Station in Brownwood Texas. Shells were significantly higher in total phenolics, condensed tannins, and antioxidant activity compared to kernel extracts. Among the shells, Kanzi, followed by Nacono, Kiowa, Pawnee, Shawnee, and Desirable were the highest in total phenolics. Similar results were reported for antioxidant assays. Following chemical assays, crude kernel and shell extracts were unable to be characterized by high performance liquid chromatography (HPLC). Crude extracts were hydrolyzed with base followed by acid, which removed the interfering components and allowed for the identification of 5 phenolic acids in kernel (gallic acid, ellagic acid, catechin, and epicatechin) and 2 phenolic acids in shells (gallic acid and ellagic acid). Cultivar did not significantly affect the content of gallic and ellagic acid identified in basic/acid hydrolyzed extracts.

Malik, Perez, Lombardini, Cornacchia, Cisneros-Zevallos, and Bradford (2009), showed that the use of a destructive pre-hydrolysis step is not required to separate and characterize phenolics in pecan. This study compared different cultivars grown conventionally or organically. Kernel extracts obtained using 80% methanol as a solvent were separated using gel chromatography with Sephadex LH-20 resin. Nine phenolic compounds identified by reverse phase HPLC, with gallic acid, catechin, and ellagic acid in quantifiable amounts. Other phenolics included catechol, epicatechin, *m*-coumaric acid, chlorogenic acid, ellagic acid, caffeic acid and ellagic acid derivative. The phenolic content of organically grown kernels were significantly higher and dependent on cultivar. This suggest that multiple factors in combination are responsible for the specific bioactive profile of pecan (Malik, Perez, Lombardini, Cornacchia, Cisneros-Zevallos, and Bradford, 2009).

Rosa, Alvarez-Parrilla, and Shahidi 2011 evaluated the effect of growing region. Acetonic extracts (80% v/v) from nut shells and kernels were obtained from pecans cultivated in North, Central, and Southern regions of Chihuahua, Mexico and analyzed by colorimetric assays. Pecan shells from the southern region were significantly higher in percent yield, total phenolics, flavonoids, and condensed tannins. This geographical affect was not observed in analyses of kernels.

2.2.3. Knowledge gap

Shifts in social perceptions about the safety of synthetic products, has catapulted a new wave of plant bioactive research. Improved separatory and spectrometric techniques have aided in the discovery, and characterization of thousands of plant bioactive components. Many of which have potential to be used as natural antimicrobials and antioxidants and nutraceuticals, among other things. There is an abundance of pecan characterization studies.

However, the kernel has overshadowed shell research. This is due to the kernel's edible nature, and status as a healthy food. Furthermore, analytical difficulties (interfering components, inefficient separation, and the use of destructive techniques) have limited shell characterization studies.

Many factors including growing region, cultivar, cultivation method, and harvest year have been shown to significantly affect the bioactive properties of pecan. Previous studies on pecan shell do little to address the influence of these factors. There is a lack of studies that extensively compare the effect of cultivar across a large population while controlling the harvest year, growing region, and cultivation method. Comparative studies on extraction method are limited, and typically only compare a single cultivar.

Louisiana is the 5th largest producer of pecans in the United States. Annual production generates nearly 6 million pounds of by-product that is underutilized. The shells of Louisiana pecan have not been studied for their potential antioxidant properties. Comparative studies on the most appropriate extraction method are lacking. This warrants the study on the effect of cultivar and extraction method on shell bioactive components of pecans cultivated in Louisiana.

2.3. References

1. Ayabe, S. -I., Uchiyama, H., Aoki, T., & Akashi T. (2010). Plant Phenolics: Phenylpropanoids. Nihon University, Fujisawa, Japan. doi: 10.1016/B978-008045382-8.00023-X

2. Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N., & Omar, A. K. M. (2013). Techniques for extraction of bioactive compounds from plant materials: A review. *J. Food Engineering*, *117*, 426-436. doi: 10.1016/j.foodeng.2013.01.014

3. Azwanida, N. N. (2015). A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength ad Limitation. *Med Aromat Plants*, *4*(3), 196-202.

doi: 10.4172/2167-0412.1000196

4. Bernhoft, A. (2010). A brief review on bioactive compounds in plants. *Bioactive compounds in plants- benefits and risks for man and animals*. Novus forlag, Oslo. Retrieved from http://www.dnva.no/binfil/download.php?tid=48677

5. Blomhoff, R. (2010). Role of dietary phytochemicals in oxidative stress. *Bioactive compounds in plants- benefits and risks for man and animals*. Novus forlag, Oslo. Retrieved from http://www.dnva.no/binfil/download.php?tid=48677

6. Cazambu. S., Biondo, E., Kolchinski, E. M., Padilha, R. L., Brandelli, A., & Santanna, V. (2016). *Food Sci. Technol*, *36*(1), 42-45. doi: 10.1590/1678-457X.0043

7. Davidson, P. M., Critzer, F. J., & Taylor, T. M. (2013). Naturally occurring antimicrobials for minimally processed foods. *Annu Rev Food Sci Technol*, *4*, 163-190. doi: 10.1146/annurev-food-030212-182535

8. Deng, Y. & Lu, S. (2017). Biosynthesis and Regulation of Phenylpropanoids in Plants. *Critical Reviews in Plant Science*, *36*(4). doi: 10.1080/07352689.2017.1402852

9. Joana Gil-Chávez, G., Villa, J. A., Fernando Ayala-Zavala, J., Basilio Heredia, J., Sepulveda, D., Yahia, E. M., & González-Aguilar, G. A. (2013). Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food Ingredients: An Overview. *Comprehensive Reviews in Food Science and Food Safety, 12*(1), 5–23. doi:10.1111/1541-4337.12005

10. Lucera, A., Costa, C., Conte, A., & Nobile, M. A. D. (2012). Food applications of natural antimicrobial compounds. *Frontiers in Microbiology*, *3* (287), 1-12. doi: 10.3389/fmicb.2012.00287

11. Malik, N., Perez, J.L., Lombardini, L., Cornacchia, R., Cisneros-Zevallos, & Braford, J. (2009). Phenolic compounds and fatty acid composition of organic and conventional grown pecan kernels. *J Sci Food Agric, 89*, 2207-2213. doi:10.1002/jsfa.3708

12. New Mexico State University (NMSU). (2005). Growth and Development of Pecan Nuts Guide H-618. Retrieved from https://aces.nmsu.edu/pubs/_h/H618.pdf

13. Nielsen. (2015). We are What We Eat Healthy Eating Trends Around the World. Retrieved from https://www.nielsen.com/content/dam/nielsenglobal/eu/nielseninsights/pdfs/Nielsen%20Global%20Health%20and%20Wellness%20Report%20-%20January%202015.pdf

14. Papuc, C., Goran, V. G., Predescu, C. N., Nicorescu, V., & Stefan, G. (2017). Plant Polyphenols as Antioxidant and Antibacterial Agents for Shelf-Life Extension of Meat and Meat Products: Classification, Structures, Sources, and Action Mechanisms. *Comprehensive reviews in Food Sci. and Food Safety*, *16*, 1243-1268. doi: 10.111/1541-4337.12298

15. Prado, A. C. P., Aragão, A. M., Fett, R., & Block, J.M. (2009). Antioxidant Properties of Pecan Nut [Carya illinoinensis (Wangenh.) C. Koch] Shell Infusion. *Grasas y Aceites*, *60* (4), 330-335. doi: 10.3989/gya.107708

16. Prado, A. C. P., Silva, H.S., Silveira, S.M., Barreto, P. L. M., Vieira C. R. W., Maraschini, M., Ferreira, A. R. S., & Block J. M. (2014). "Effect of the extraction process on the phenolic compounds profile and the antioxidant and antimicrobial activity of extracts of pecan nut [Carya illinoinensis (Wangenh) C. Koch] shell." *Industrial Crops and Products, 52*, 552-561. doi: 10.1016/j.indcrop.2013.11.031

17. Rosa, L. A., Alvarez-Parrilla, E., & Shahidi, F. (2011). Phenolic Compounds and Antioxidant Activity of Kernels and Shells of Mexican Pecan (*Carya illinoinensis*). *J. Agric. Food Chem.*, *59*, 152-162. doi: 10.1021/jf1034306

18. Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Yoga, L. L. (2011). Extraction, Isolation and Characterization of Bioactive Compounds from Pants' Extracts. *Afr J Tradit Complement Altern Med*., *8* (1), 1-10.

19. Selma, M. V., Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between Phenolics and Gut Microbiota: Role in Human Health. *Journal of Agricultural and Food Chemistry*. *57*. 6485-501. 10.1021/jf902107d.

20. Smith, R. M. (2003). Before the injection-modern methods of sample preparation for separation techniques. *J. of Chromatography A*, *1000*, 3-27. doi: 10.1016 /S0021-9673(03)00511-9

21. Snyder, L. R. (1974). Classification of the solvent properties of common liquids. *Journal of Chromatography A.*, *92* (2), 223-230. doi: 10.1016/s0021-9673(00)85732-5

22. Soobrattee, M. A., Neergheen, V.S., Luximon-Ramma, A., Aruoma, O. I., & Bahorun, T. (2005). Phenolics as potential therapeutic agents: Mechanism and actions. *Fundamental and Molecular Mechanisms of Mutagenesis*, *579*, 200-213. doi: 10.1016/j.mrfmmm.2005.03.023

23. United States Department of Agriculture Economic Research Service (ERS). (2017). Organic Market Overview. Retrieved from

https://www.ers.usda.gov/topics/natural-resources-environment/organic-agriculture/organic-market-overview.aspx

24. United States Department of Agriculture National Agriculture Statistics Service (NASS). (2018). National Statistics for Pecan. Retrieved from https://www.nass.usda.gov/Statistics_by_Subject/result.php?7345CB67-26CC3917AF5BC7828216D668&sector=CROPS&group=FRUIT%20%26%20TREE%20NUTS&comm=PECANS

25. Villarreal-Lozoya, J. E., Lombardini, L., & Cisneros-Zevallos, L. (2007). Phytochemical constituents and antioxidant capacity of different pecan [*Carya illinoinensis* (Wangenh.) K. Koch] cultivars. *Food Chem., 102*, 1241-1249. doi: 10.1016/j.foodchem.2006.07.024

26. Vladimir-Knežević, S., Blažeković, B., Štefan M. B., & Babac, M. (2012). Plant Polyphenols as Antioxidants Influencing the Human Health, Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health, Venketeshwer Rao, IntechOpen, doi: 10.5772/27843

3. ANTIOXIDANT PROPERTIES OF PECAN SHELL BIOACTIVE COMPONENTS OF DIFFERENT CULTIVARS AND EXTRACTION METHODS

3.1. Introduction

Pecan nuts are a highly valued but underutilized crop. Over 270 million pounds are produced annually in the United States. Following harvest, over 90% of pecan nuts are processed to remove the outer shell layer, and only the edible kernel is sold for consumption (NASS, 2018). The shell by-product constituents approximately 50% of the harvested mass (Worley, 1994). As it stands, they provide very little to no revenue for pecan shellers and can be a significant disposal issue.

The natural foods sector has undergone significant growth over the past decade (Statista, 2019). This is partly due to consumers consciousness about potential health risks associated with synthetic ingredients. In response, demands are shifting away from food products preserved by conventional chemical or physical methods, in favor of "natural" or organic products (ERS, 2017). Food products under this category can be charged for a higher price but pose new technical challenges in terms of shelf stability. Recently plant bioactive compounds have gained attention for their functional properties. Several studies have determined pecan shells to be a rich source of phenolic compounds ranging from phenolic acids, flavan-3-ols, and anthocyanins (Villareal-Lozoya, Lombardini, & Cisneros-Zevallos, 2007; Prado et al., 2014; Rosa et al., 2014). These compounds are known to have antioxidant and antimicrobial properties . Thus, pecan shells have potential to be used as an alternative source of natural antioxidants in various food applications. Many factors such as growing region (Rosa, Alvarez-Parrilla, & Shahidi 2011; Rosa et al., 2014), cultivar (Villareal-Lozoya, Lombardini, & Cisneros-Zevallos, 2007; Prado, Aragão, Fett, & Block, 2009), cultivation

method (Malik, Perez, Lombardini, Cornacchia, Cisneros-Zevallos, & Braford, 2009), and harvest year (Prado et al., 2013) have been shown to significantly affect the bioactive profile and antioxidant activity of pecan components. The antioxidant activity of extractable shell bioactive components of Louisiana pecan cultivars has not been studied. Furthermore, there is a lack of comparative studies on extraction methods to obtain extracts with highest antioxidant efficiency.

The main objectives of this study are to: (i) To evaluate the effect of cultivar on the phenolic content and antioxidant activity of shell extracts of pecans grown in Louisiana, United States, (ii) To determine the effect of extraction method on the phenolic content and antioxidant activity of pecan shell bioactive components, and (iii) Characterize the bioactive components in pecan shell extracts.

3.2. Materials and methods

3.2.1. Chemicals and reagents

All reagents and standards that were used in chemical assays were ACS grade. Folin-Ciocalteu phenol reagent, DPPH (2, 2-Diphenyl-1-picrylhydrazyl), gallic acid (3, 4, 5-Trihydroxybenzoic acid), trolox (6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), sodium carbonate monohydrate, ACS grade solvents hexane, methanol, and hydrochloric acid, as well as HPLC grade acetic acid, and acetonitrile were purchased from VWR International (Radnor, PA, USA). University Stores (Baton Rouge, LA, USA) supplied ethanol (95% v/v). Sephadex™ LH-20 resin was purchased from GE Healthcare Life Sciences (Marlborough, MA, USA).

3.2.2. Pecan cultivars

Louisiana State University AgCenter Pecan Research and Extension Station, Shreveport LA, United States provided in-shell pecan nuts [*Carya illinoinensis* (Wangenh) C. Koch]. Pecans used in this study were harvested in September through November 2017, after having received all the same fertilization and pesticide applications. The pecans were sampled from trees of different ages from three different orchards. Cape Fear, Creek, Gloria Grande, Jackson, Maramec, and Melrose cultivars were grown in the *Northwest* orchard that was established in 1981. Cherokee, Curtis, Kiowa, Moreland, Point Coupee, Schley, Success, and Sumner were harvested from the *Pathology* orchard that was established in 1988. The youngest orchard sampled was the *Demo* orchard, having provided nuts from trees planted in 2005.

3.2.3. Sample preparation

Pecans were stored in a refrigerated storage room (4^{o}C) upon arrival to Louisiana State University. Pecans from 20 cultivars were removed from refrigerated storage, individually cracked using a nutcracker, and the shells were then separated. Later, the shells were crushed to a smaller size before being dried in a convection oven (VWR ShelLab Model 1370 GM) for 8 hours at 40^{o}C. Dried nut shells were grinded into a powder using a food processor (Magic Bullet MB1001C). The resulting dried pecan nut shell powder (NSP) was stored in 250 ml amber colored glass bottles at -19^{o}C for future use. NSP from each cultivar was transferred from cold storage and allowed to equilibrate to 23^{o}C. Solid-liquid extraction was used to remove lipids from pecan shells. NSP from each cultivar (8 g) was individually weighed and placed in a new 250 ml amber colored bottle. A volume of 160 ml of hexane (1:20 W/V) was added to each bottle and then thoroughly mixed at 160 rpm using New

Brunswick Scientific C25KC Incubator Shaker for a period of 45 min at 22°C. Hexane was then slowly filtered from the pecan shell residue using a Buchner funnel equipped with a filter paper (Whatman no. 1) under vacuum. This process was repeated twice, and the defatted pecan nut shell powder cakes were placed inside a chemical hood for 4 hours in the absence of light to allow residual hexane solvent to evaporate. Defatted sample were stored in 250 ml amber colored bottles in the absence of light at -19°C.

3.2.4. Bioactive extraction

Bioactive compounds from defatted pecan NSP were isolated by solid-liquid extraction using either distilled water or ethanol (95% v/v) as a solvent. On the morning of extraction, defatted NSP was removed from the freezer (-20°C) and allowed to equilibrate to 23°C. To perform aqueous extractions, a 2 g aliquot of defatted NSP from each cultivar was weighed (Mettler Toledo XS204) and placed into individual 250 ml amber bottles. Aqueous infusions (20 g/L) were prepared by pouring 160 ml distilled water at 98°C into each 250 ml amber bottle containing NSP, quickly the bottles were capped and placed in a Buchi 461 hot-water bath (98°C) during 30min, with mixing every 5 min. Following extraction, aqueous infusions were removed from the hot water bath and allowed to cool for 10 min. Extracts were then filtered under vacuum using a Buchner funnel equipped with a filter paper (Whatman no. 1). The extracts were collected in individual 250 ml amber bottle and the pecan shell residue was re-extracted following the same procedure. The extracts from the first and second aqueous fractions were combined and stored in -80°C freezer. Ethanolic extracts were prepared by mixing defatted NSP with ethanol (20 g/L) in 250 ml amber colored bottles and were constantly mixed at 160 rpm using New Brunswick Scientific C25KC Incubator Shaker for 60min at 22^{0}C. Then, the extracts were filtered as previously

described and stored at -80^{0}C. Extracts were concentrated to a powder by lyophilization. Prior to chemical analysis, aliquots of lyophilized extracts were diluted in methanol 0.2 mg/ml, vortexed, and filtered (0.45 µm).

3.2.5. Determination of phenolic content

Total phenolic content of pecan nut shell aqueous and ethanolic extracts was estimated by the Folin-Ciocalteu colorimetric assay using microtiter plate according to Singleton, Orthofer, and Lamuela-Raventos 1999. In a 96-well microplate, 30 µL aliquots of each freeze-dried diluted extracts were mixed with 150 µL of Folin-Ciocalteu reagent (1:10, v/v in distilled water). After 5 minutes, the reaction was neutralized with 120 µL sodium carbonate (75 g/L) and then incubated at 22°C for 90 minutes in the dark. The absorbance of the resulting reactions was measured via microplate reader (Bio-Rad Benchmark Plus) at 765nm. A Gallic acid standard curve (300, 250, 200, 150, 100, 75, 50, 25 µg/ml) was generated as a reference, therefore data were expressed as mg gallic acid equivalents per gram freeze dried extract (mg GAEg^{-1}). Analysis were carried out in duplicates with three replications in each.

3.2.6. Evaluation of antioxidant activity

The evaluation of the antioxidant potential of shell extracts was conducted via DPPH [2, 20-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] free radical assay as described by Brand-William, Cuvelier, and Berset 1994, with some modifications. In a microplate, a 10 µL aliquot of diluted extracts was reacted with 200 µL of DPPH (.01 M DPPH in methanol), the plate was covered and incubated in the absence of light at 22°C for 30 min. A microplate reader (Eppendorf AF2200) was then used to measure the initial and final absorbance at 540nm. Radical scavenging activity was calculated according to the following equation:

$$\text{Radical scavenging effect (\%)} = \frac{(A_{540}\ 0\ \text{min} - A_{540}\ 30\ \text{min})}{A_{540}} x\ 100$$

A Trolox standard curve (500, 250, 200, 100, 50, 25, 10 µg/ml) was generated to quantify antioxidant activity of the extracts. Results were expressed as mg Trolox equivalents per gram freeze dried extract (mg TEg^{-1}). Analysis were carried out in duplicates with three replications each.

3.2.7. Reverse phase high performance liquid chromatography (RP-HPLC)

Following pre-screening of extracts from all 20 cultivars for total phenolics and antioxidant activity, aqueous and ethanolic extracts from one high (Caddo) and one low (Nacono) performing cultivars were selected for chemical profiling. RP-HPLC with UV/VIS absorbance detection was used to characterize crude extracts and acid hydrolyzed extracts. Acid hydrolysis was performed to free glyosidic bound phenolic compounds. Crude extracts were weighed and placed in a 250 ml amber bottles. Acidified methanol (1% HCl v/v) for 24 h at 23°C under constant shaking 160 rpm. The resulting extracts were centrifuged at 6,500g for 6 minutes and the resulting supernatant was dried using a Labconco 7812013 Centrivap evaporator at 70°C. Extracts were diluted in methanol (25 mg/ml) and then centrifuged at 12,000 x g during 10 min to remove insoluble material and then transferred to a 1.5 ml vial for analysis. Chromatographic separations of extracts were performed using a Waters Alliance 2690 HPLC system equipped with a 996-photodiode array detector. Bioactive compounds were separated using 4.6mm x 250mm C18 column. A 50 µL volume of extract was eluted in a bi-solvent mobile phase composed of aqueous acetic acid (10% v/v) (solvent A) and acetonitrile (solvent B) for a total run time of 94 minutes. Prior to samples injection the column was equilibrated with 100% solvent A. Upon injection, the samples were eluted

at a flow rate of 0.8 ml min^{-1} with the following gradient: A 100% for 0-50 min, A 70% and B 30% 50-70 min, A 50% and B 50% 70-80 min, A 20% and B 80% 80-85 min, B 100% 85-90 min, and A 100% 90-94 min.

3.2.8. LH-20 Sephadex column chromatography

Crude Caddo ethanolic extracts were separated using a lipophilic resin (LH-20) according to Malik, Perez, Lombardini, Cornacchia, Cisneros-Zevallos, & Braford 2009. To prepare the column, Lipophilic GE Sephadex™ LH-20 resin (.750 gm) was conditioned with 3.75 ml aqueous methanol (80% V/V) in a 15 ml tube for 24h at room temperature. The resulting slurry was gently mixed and then slowly transferred using a graduated pipette into a 10 mm x 100 mm Omnifit EZ glass column until the resin bed was packed to the 10 cm line. When the 10 cm line was reached, mobile phase (80% aqueous methanol) was added to cover the top of the resin bed and the column was sealed until further use. Crude Caddo ethanolic extract was weighed on analytical balance and placed in a micro-centrifuge tube. The extract was diluted with 1 ml mobile phase (50 mg/ml) and vortexed to mix. After mixing, diluted extract was filtered through a 0.45 μm filter. The column bed was washed by passing 10 ml mobile phase at flow rate of 0.5 mL min^{-1} using a Bio-Rad Econo Gradient Pump prior to extract elucidation. Aliquots of 300 μL of diluted extract were gently loaded on top of the LH-20 column bed with a micro-pipette and eluted with 10 ml of 80% aqueous ethanol at 0.5 mL min^{-1} and five 2 ml fractions were collected in 15 ml centrifuge tubes using a Waters fraction collector (WFC 43030). The column was washed with 10 ml mobile phase after the 5 fractions were collected and before the next sample was injected. This process was repeated 2 times for a total of 3 injections. Similar extract fractions were mixed together and analyzed with a VWR UV-3100PC UV/VIS scanning spectrophotometer. Absorbance

spectrums were collected from 800nm to 240nm at a scan rate of 5 nm/s. An absorbance spectrum signal from the mobile phase was collected as a reference blank and was subtracted from the signals collected for the 5 fractions. An absorbance spectrum of Gallic acid was collected and used as a free phenolic reference.

3.2.9. FIA-ESI-MS

Flow injection analysis mass spectrometry using an Advion expression^{L} CMS mass spectrometer was performed on acid hydrolyzed Nacono ethanolic extracts to confirm potential compounds identified using RP-HPLC-DAD. A 5 µL volume of extract was manually injected and ionized with either electrospray ionization (ESI) with a typical fragmentation setting with acetonitrile (75% v/v) as a mobile phase. Positive and negative ions from 50-1200amu were recorded in the mass spectrums. Background noise was collected and subtracted from the total ion count chromatograms.

3.2.10. Statistical model

The effect of extraction method was evaluated under the assumptions that total phenolic content (TPC) or free-radical scavenging activity of aqueous and ethanolic pecan shell extracts from corresponding cultivars were equal (H_0: $\mu_{aqueous} = \mu_{ethanolic}$). The claim that either TP or DPPH of ethanolic and aqueous extracts from corresponding pecan cultivars were different was tested using a two-sided paired t-test ($P \leq 0.05$) on replication means (Ha: $\mu_{aqueous} \neq \mu_{ethanolic}$). This t-test is appropriate for our data set because it allows you to determine if a difference exists between two values that correspond to a common group. In our analysis we are comparing either total phenolics or antioxidant activity of the extractions obtained by two different extraction methods on a common cultivar. The effect of cultivar on the phenolic content and antioxidant activity of ethanol and aqueous extracts was evaluated

using a two sperate one-way analysis of variances (ANOVA), with a post hoc Tukey (HSD) test ($P \leq 0.05$).

3.3. Results and discussion

3.3.1. Effect of extraction method on total phenolic and antioxidant activity

The antioxidant properties of pecan have been attributed to phenolic compounds. The Folin-Ciocalteu colorimetric assay is widely used to estimate the total phenolic content (TPC) of plant extracts. However, this method is not specific for phenolic compounds, and is sensitive to other reducing agents. The DPPH free radical scavenging assay is used to measure the ability of a plant extracts ability to annihilate radicals, or in other word, retard free-radical initiated oxidation. Together, these assays are an indicator of total relative antioxidant potential (Sánchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velázquez 2013). Chun & Kim 2014 showed that monomeric phenolics were more reactive to Folin-Ciocalteu reagent compared to their multimeric derivates. Soobrattee, Neergheen, Luximon-Ramma, Aruoma, and Bahorun, 2005 suggests that higher degrees of flavonoid polymerization predict an increase in antioxidant activity measured by DPPH assay. TPC and free-radical scavenging activity of pecan shell extracts from 20 cultivars obtained by either aqueous or ethanol solid-liquid extraction is reported in Table 3.1 Method of extraction significantly ($P \leq 0.05$) affected TPC of crude extracts estimated using the Folin-Ciocalteu assay. The TPC of ethanolic extracts ranged from 304.18 to 153.54 mg $GAEg^{-1}$ dry extract with an average of 210.02±7.3 mg $GAEg^{-1}$ and were significantly greater ($P < 0.05$) than those obtained by aqueous extraction, which ranged from 253.75 to 114.63, with an average of 168.38±6.8 mg $GAEg^{-1}$ of dry extract.

Table 3.1. Antioxidant activity assays of pecan bioactive components from twenty pecan cultivars extracted by either distilled water or ethanol solid-liquid extraction

Cultivar	TPC[A] (mg GAEg^{-1} dry extract)		DPPH[B] (mg TEg^{-1} dry extract)	
	Aqueous extracts	Ethanolic extracts	Aqueous extracts	Ethanolic extracts
Desirable[C]	167.0^{bcde}	209.8^{xyz}	690.6^{ab}	611.9^{wxyz}
Caddo	176.8^{becd}	212.2^{xyz}	600.6^{b}	680.4^{vwxyz}
Elliot	130.7^{fe}	234.9^{xyz}	468.3^{b}	768.2^{vwxy}
Nacono	174.1^{bcde}	179.2^{yz}	574.2^{b}	580.2^{xyz}
Oconee	175.7^{bcde}	183.3^{yz}	599.7^{b}	571.1^{xyz}
Pawnee	202.4^{ab}	195.5^{yz}	666.5^{ab}	608.4^{wxyz}
Point Coupee[D]	189.5^{bcd}	304.2^{x}	612.6^{b}	796.1^{vwx}
Curtis	253.8^{a}	209.9^{yz}	934.9^{a}	820.4^{vw}
Kiowa	173.3^{bcde}	190.2^{xyz}	656.8^{b}	581.7^{xyz}
Moreland	150.4^{bcdef}	215.4^{xyz}	718.6^{ab}	630.8^{vwxyz}
Cherokee	165.2^{bcdef}	153.5^{z}	630.2^{b}	652.9^{vwxyz}
Schley	197.3^{bc}	194.1^{yz}	667.4^{ab}	547.5^{yz}
Success	167.45^{bcde}	173.6^{yz}	606.3^{b}	542.5^{z}
Sumner	175.6^{bcde}	195.5^{yz}	569.6^{b}	544.3^{z}
Gloria Grande[E]	149.8^{fecd}	231.6^{xyz}	630.5^{b}	733.0^{vwxyz}
Cape Fear	143.2^{def}	203.4^{yz}	606.4^{b}	526.7^{z}
Creek	149.5^{cdef}	202.8^{yz}	638.7^{b}	650.5^{vwxyz}
Maramec	184.2^{bcd}	263.2^{xy}	522.6^{b}	840.6^{v}
Jackson	114.7^{f}	227.9^{xyz}	538.1^{b}	710.5^{vwxyz}
Melrose	126.0^{ef}	220.3^{xyz}	495.3^{b}	668.4^{vwxyz}
Average ± SE	168±6.8	210±7.3	659±21	619±22

[A] Total extractable phenolic content (Folin-Ciocalteu assay) expressed in mg gallic acid equivalents per gram of free-dried extract.

[B] Free-radical scavenging activity (DPPH assay) express in mg trolox equivalents per gram of freeze-dried extract.

[C] Demo orchard – 2005 est.

[D] Pathology orchard – 1988 est.

[E] Northwest orchard – 1981 est.

Values in a column that share a lower-case letter are not significantly different (P<0.05)

The TPC of ethanolic extracts ranged from 304.18 to 153.54 mg $GAEg^{-1}$ dry extract with an average of 210.02±7.3 mg $GAEg^{-1}$ and were significantly greater (P<0.05) than those obtained by aqueous extraction, which ranged from 253.75 to 114.63, with an average of 168.38±6.8 mg $GAEg^{-1}$ of dry extract. However, method of extraction did not significantly affect free-radical scavenging activity measured by the DPPH assay. Free-radical scavenging activity of ethanolic extracts ranged from 820.39 to 526.74 and averaged 659.70±21 mg TEg^{-1}, while aqueous extracts ranged from 934.95 to 468.34 with an average of 619.42±22 mg TEg^{-1}.

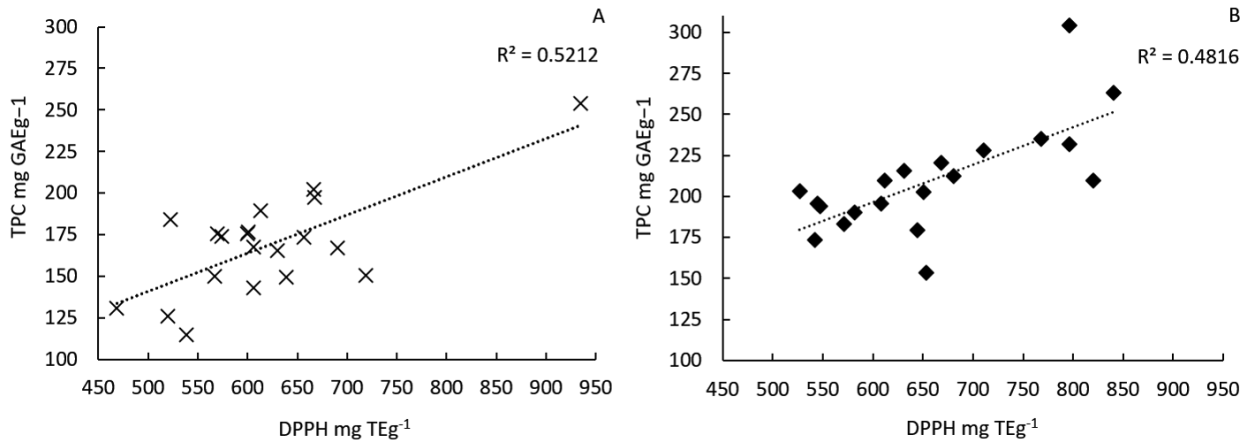

Figure 3.1. Positive Linear correlation between the total phenolic content (TPC) reported as mg $GAEg^{-1}$ (gallic acid equivalents per gram dry extract) measured using the Folin-Ciocalteu assay and antioxidant activity measured by the DPPH method reported in mg TEg^{-1} (Trolox equivalents per gram dry extract) of 20 pecan cultivars extracted by distilled water (20 g/L, 30 min, 22°C) (A) or ethanol (20 g/L, 1 hr, 22°C) (B) solid-liquid extraction.

A positive linear correlation between phenolic content and antioxidant activity was observed for aqueous (R^2=0.52) and ethanolic extracts (R^2=0.48) (Figure 1). Pecan shell aqueous infusions were found to have a much stronger linear relationship (R^2=0.99) when extracts were not dried prior to analysis (Prado, Aragão, Fett, & Block, 2009). Villareal-Lozoya, Lombardini, & Cisneros-Zevallos 2007 reported that phenolic content and

antioxidant activity were more closely associated (R^2=0.61) in acetone: water (70:30 v/v) extracts than from extracts obtained in this study.

In disagreement with this study Prado, Aragão, Fett, & Block 2009 reported that TPC (181.49±6.97 mg $GAEg^{-1}$) and antioxidant activity of aqueous extracts (DPPH 612.24±26.73 mg TEg^{-1}, ABTS 1809.01±27.18 mg Teg^{-1}) was significantly greater than ethanol extracts (167.85±3.89 mg $GAEg^{-1}$, DPPH 524.77±40.72 mg Teg^{-1}, ABTS 1562.51±33.15 mg Teg^{-1}). High gallic acid and epigallocatechin gallate content was strongly associated with high antioxidant activity measured by the DPPH assay. Interestingly, the condensed tannin content of ethanol extracts was 11 times greater than aqueous extracts (Prado et al., 2014).

In a study by Villareal-Lozoya, Lombardini, & Cisneros-Zevallos 2007 water: acetone (70:30 v/v) extracts from various cultivars averaged 448±45 mg $CAEg^{-1}$ with catechin (monomer) as a reference compound. It is difficult to directly compare the estimated phenolic content of similar cultivars in this study, and Villareal-Lozoya, Lombardini, & Cisneros-Zevallos 2007 due to the use of different reference standards. In agreement with Prado et al. 2014, Villareal-Lozoya, Lombardini, & Cisneros-Zevallos 2007 reported that acetone: water (70:30) extracts from defatted shells contained 10-23 times greater condensed tannin content compared to aqueous extracts. This indicates that extraction efficiency of condensed tannins is increased when an organic solvent is used.

The observed variation between phenolic content and antioxidant activity of extracts of different extraction methods in this study and past works may be caused by other factors. Pecans are affected by alternate bearing cycles, in which carbohydrate storage is significantly reduced. As a result, the health and reproduction capabilities of the tree are negatively impacted. Thus, affecting their nut bioactive profile. Malik, Perez, Lombardini,

Cornacchia, Cisneros-Zevallos, and Braford 2009 reported significant differences in phenolic content between pecans produced by different cultivar methods. Geographical location also may affect the phenolic profile of kernel and nutshells (Rosa et al., 2014).

3.3.2. Effect of cultivar on the phenolic content and free-radical scavenging activity

Extensive breeding efforts in the United States between 1960 and 1980, has led to the development of over 500 pecan cultivars. Cultivars commonly called "improved varieties" are breed to more resistant to environmental stresses and produce nuts with thin shell walls and kernels that are high and lipid and resist oxidation over long storage times (Worley, 1994). Stress adaptation is an action of secondary bioactive components produced by the plant. It has been suggested, shell phenolics and antioxidant activity is higher in cultivars with kernels containing high amounts of lipids (Prado et al., 2013).

Pecan cultivar significantly ($P \leq 0.05$) affected both the TPC and free-radical scavenging activity of aqueous and ethanolic extracts. When considering the aqueous extracts, the tested cultivars ranked from highest to lowest TPC are as follows: Curtis ≥ Pawnee ≥ Schley ≥ Point-Coupee ≈ Maramec ≥ Caddo ≈ Oconee ≈ Sumner ≈ Nacono ≈ Kiowa ≈ Success ≈ Desirable ≥ Cherokee ≈ Moreland ≥ Gloria Grande ≈ Creek ≥ Cape Fear ≥ Elliot ≥ Melrose > Jackson. Free radical scavenging activity of aqueous extracts followed the trend: Curtis ≥ Moreland ≈ Desirable ≈ Schley ≈ Pawnee ≥ Kiowa ≈ Creek ≈ Cherokee ≈ Point Coupee ≈ Cape Fear ≈ Success ≈ Caddo ≈ Oconee ≈ Nacono ≈ Sumner ≈ Gloria Grande ≈ Jackson ≈ Maramec ≈ Melrose ≈ Elliot. Prado, Aragão, Fett, & Block, 2009 reported that aqueous shell extracts from a mixture of Barton (approximately 50%), Shashone, Shawnee, Choctaw, and Cape Fear were lower in phenolic content (138±26 mg $GAEg^{-1}$) and antioxidant activity (572±102 mg $TEACg^{-1}$) compared to respective averages for aqueous extracts in this study. In this study methanol

soluble components of aqueous extracts were quantified in methanol for chemical assays, while Prado et al. 2009 assayed extracts in aqueous solution. TPC of ethanolic extracts followed the trend: Point-Coupee > Maramec ≥ Elliot ≈ Gloria Grande ≈ Jackson ≈ Melrose ≈ Moreland ≈ Caddo ≈ Desirable ≥ Curtis ≈ Cape Fear ≈ Creek ≈ Pawnee ≈ Sumner ≈ Schley ≈ Kiowa ≈ Oconee ≈ Nacono ≈ Success > Cherokee. The antioxidant activity of extracts by ethanol extraction followed the trend: Maramec ≥ Curtis ≥ Point Coupee ≥ Elliot ≥ Gloria Grande ≈ Jackson ≈ Caddo ≈ Melrose ≈ Cherokee ≈ Creek ≈ Moreland ≥ Desirable ≈ Pawnee ≥ Kiowa ≈ Nacono ≈ Oconee ≥ Schley ≥ Sumner ≈ Success ≈Cape Fear.

Villareal-Lozoya, Lombardini, & Cisneros-Zevallos 2007 showed that cultivar significantly affected (Tukey, $P<0.05$) the total phenolic content (TPC)(Folin-Ciocalteu assay) and antioxidant capacity (DPPH assay) of dried shell extracts obtained using acetone: water (70:30 v/v) as a solvent from 6 different cultivars that were harvested from the same orchard in 2007. The Kanza (TPC 633±29 mg $CAEg^{-1}$, DPPH 675±18 mg TEg^{-1}) followed by Pawnee (TPC 537±10 mg $CAEg^{-1}$, DPPH 582±29 mg TEg^{-1}) had the greatest phenolic content and antioxidant activity. Other cultivars studied included, Shawnee (TPC 537±10 mg $CAEg^{-1}$, DPPH 444±3 mg TEg^{-1}), Nacono (TPC 451±6 mg $CAEg^{-1}$, DPPH 442±7 mg TEg^{-1}), Desirable (TPC 378±17 mg $CAEg^{-1}$, DPPH 482±30mg TEg^{-1}, and Kiowa (TPC 344±10 mg $CAEg^{-1}$, DPPH 331±11). Interestingly, when comparing common cultivars tested in the present study and Villareal-Lozoya, Lombardini, & Cisneros-Zevallos 2007. Pawnee was found to be significantly greater in phenolic content compared to the Nacono, Desirable, and Kiowa cultivars in extracts obtained with water and acetone: water (70:30 v/v) as extraction solvents. Furthermore, Nacono extracts had a higher phenolic content compared to Desirable and Kiowa cultivars when these solvents were used.

It is concluded that the phenolic and antioxidant properties of pecan shell components are dependent on numerous factors in combination. Pecans cultivated in Louisiana were found to be rich in antioxidant components. Ethanol was found to be better than distilled water as a solvent to extract phenolics from pecan shell. The antioxidant activity of extracts obtained through distilled water or ethanol extraction was highly dependent on cultivar.

3.3.3. Bioactive profile by RP-HPLC

Bioactive components in crude aqueous and ethanol extracts of Nacono and Caddo cultivars where analyzed by reverse phase HPLC with uv/vis detection using a photodiode array detector. Retention times and absorption wavelengths of eluted components where compared to phenolic standards analyzed under similar conditions to presumably characterize the extracts. Methanol soluble components of crude shell extracts were eluted from the separatory column between 5.7 and 14.4 min (aqueous) and between 5.7 and 13.3 min (ethanol), in unresolved peaks with absorption bands between 280 and 460nm (Figure 2). The most abundant peak in either extract, eluted at approximately 5.7 min with a peak area of 1.20 $x10^8$ and $1.28x10^8$ in aqueous and ethanol extracts, respectively. However, components comparable to free phenolic standards were not resolved in the broad-shouldered peak of the crude extract chromatograms. Absorption in the ultraviolent and visible regions indicates a degree of aromaticity or conjugated double bonds. Specifically, absorption bands at 280nm is associated with phenolic compounds and some amino acid structures namely tyrosine and tryptophan. The component that gives the extracts a red hue, is likely responsible for absorption at 460 nm.

Prado et al. 2013 reported that aqueous soluble shell components could be quantified by measuring absorbance at 420nm. Furthermore, the authors determined through principal

component analysis that deeper red color was associated with increased antioxidant activity and quantity of phenolics, protein, and fiber of aqueous shell extracts.

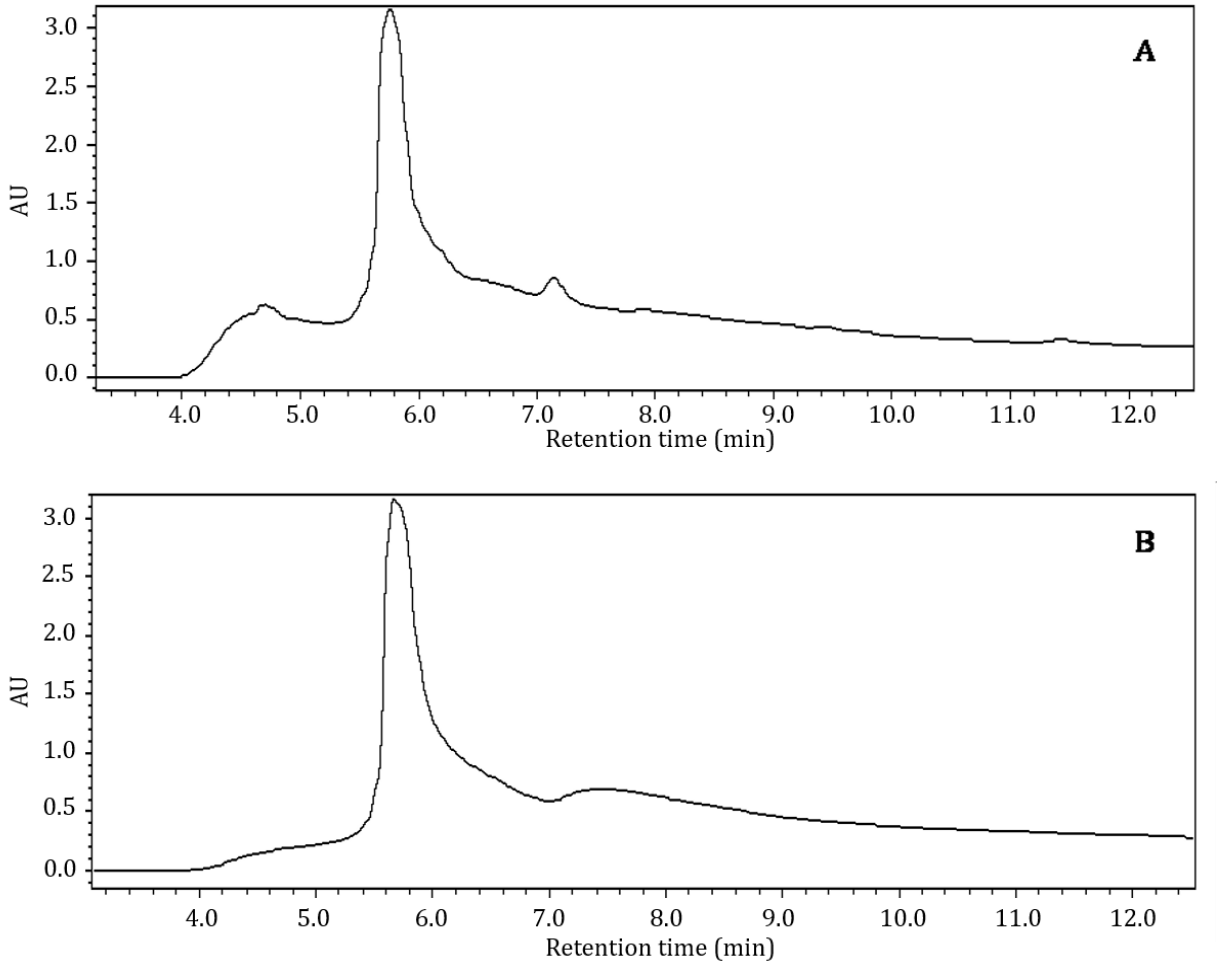

Figure 3.2. Hyphenated chromatograms (3.5-12.2 min) by HPLC of crude pecan shell Nacono extracts. A 50 µL volume of methanol soluble components were injected into a c18 column, eluted with a binary mobile phase, and were detected with a uv/vis diode array absorbance detector. The chromatograms represent detection at the 280nm wavelength channel. Pecan shells were subjected to solid-liquid extraction (20 g^{-L}) with distilled water at 98°C for 1 h (A) or ethanol at 22°C for 1 h (B) under constant mixing.

The variation in absorbance peaks between the red components in the present study, are likely due to the solvents used in the assays. Other studies have reported similar analytical challenges when characterizing phenolic components in crude extracts from pecan shell and

kernel by RP-HPLC (Villareal-Lozoya, Lombardini, & Cisneros-Zevallos, 2007; Prado et al., 2014; Rosa, Alvarez-Parrilla, & Shahidi, 2011).

Different techniques and some in combination have been used to separate pecan shell and kernel phenolic components. The most frequently employed techniques are gel-chromatography using a lipophilic Sephadex LH-20 resin, acid hydrolysis, base/acid hydrolysis, or enzymatic hydrolysis. Malik, Perez, Lombardini, Cornacchia, Cisneros-Zevallos, and Bradford 2009 and Prado et al. 2014 showed that pecan phenolic components could be sufficiently separated by Sephadex LH-20 chromatography and identified by RP-HPLC without the use of a pre-hydrolysis step. This non-destructive separatory technique is more appropriate than hydrolysis techniques when studying the native structure of an analyte. In the present study, crude shell extracts were fractionated by lipophilic Sephadex™ LH-20 Gel chromatography to further resolve pecan extracts components.

Crude Nacono ethanolic extracts were separated into 5 fractions that were analyzed by uv/vis absorbance spectroscopy. The resulting absorbance spectrums (800-200nm) were compared against a gallic acid spiked sample. Observable peaks in all fractions ranged from 290 nm to the end of the recorded spectrum (200 nm). Fraction 3, followed by 2, then 1, had the highest absorbance intensity for all observable peaks. Fractions 3 and 2 were distinguishable from other fractions by an intense peak at 280 nm. It is also worth noting that most red color pigment in the extract was never eluted from the column. This was confirmed by visually inspecting the LH-20 column following extract elucidation, and absorbance spectrums between 420-480 nm of the collected fractions. It is hypothesized that this component is of a high molecular weight and would require elution with acetone: water (1:1 v/v) (Rosa et al., 2014; Vazquez-flores et al., 2017). In the present study, aqueous

extracts were not separated due to analytical difficulties. Bioactive components in Nacono shell ethanolic extract were partially separated by LH-20 chromatography in accordance to their degree of polymerization or relative size. Absorbance spectrums did not resemble the gallic acid spiked reference, which had a distinct peak centered at 310nm (A1). Other studies have reported separation of pecan shell phenolic components by their degree of polymerization. Lerma-Herrera et al. 2017 reported that condensed tannins of varying degrees of polymerization were the major components in a shell extracts from pecan. High molecular weight fractions of shell extracts absorbed 280nm light with decreasing absorptivity as molecular weight decreased. Prado et al. 2014 separated ethanol and aqueous soluble phenolic components of defatted pecan shell by their degree of polymerization using a mobile phase of 80% ethanol. Gallic acid, chlorogenic acid, and epigallocatechin were identified in fraction 1, and their esterified form as epicatechingallate was abundant in fraction 2 of ethanolic extracts from pecan. Aqueous extract fraction 1 contained significantly higher amounts of epigallocatechin, gallic acid, and chlorogenic acid than other fractions (Prado et al., 2014).

Many studies have suggested pecan shell phenolics are primarily in oligomeric or bound forms as condensed or hydrolysable tannins or as glycosides. The use of extraction or analytical preparatory steps alters the native state of compounds, often resulting in the loss of important structural information. Furthermore, many of the structures elucidated using these techniques may be a product of the analytical methods used to extract and analyze the components of interest. Hydrolysable tannins yield gallic and ellagic acid under weak acidic or basic conditions. Oxidative cleavage of condensed tannins (proanthocyanidans) with acid yields anthocyanidin pigments and phlobaphene's associated with a red color.

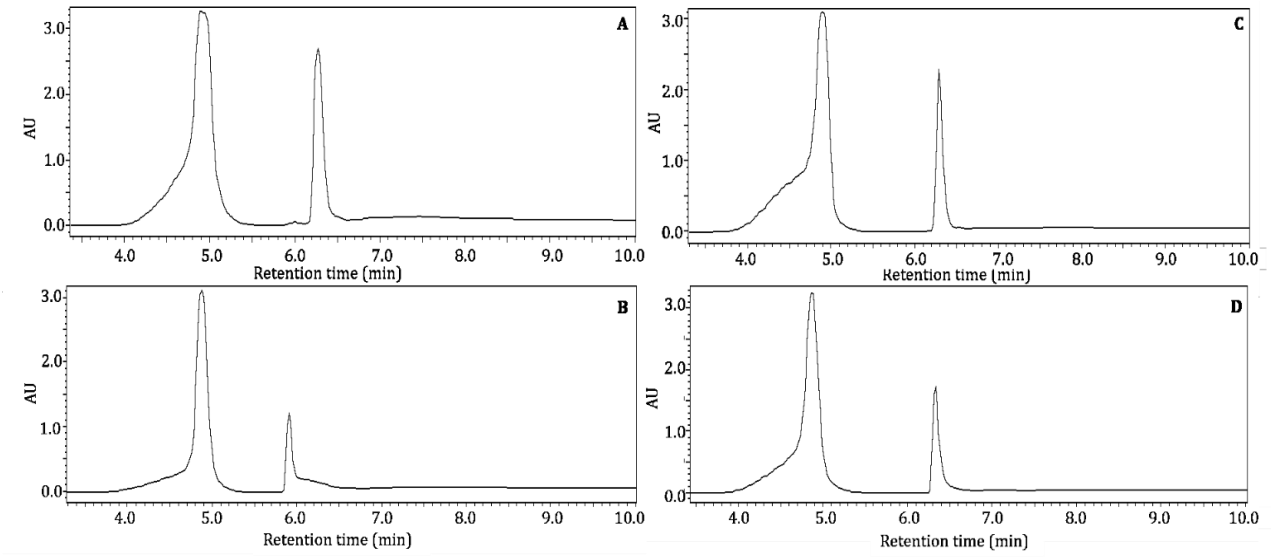

Figure 3.3. Hyphenated chromatograms from 3.5 to 10.0 min of acidified methanol (1% HCl v/v) soluble components of crude extracts analyzed by reversed phase HPLC with detection at 280nm as described in figure 2. Caddo aqueous extracts are represented by the letter A and Caddo ethanol extracts are labeled letter B. The letter C chromatogram corresponds to Nacono aqueous extract; thus, letter D represents Nacono ethanol extracts.

The treatment of lignocellulose with dilute acid in a polar solvent cleaves ester and ether linkages to produce free monomeric phenols (Hagerman, 2002). Furthermore, cleaved ester and ether bonds can reassociation into more complex polymeric structures. These modifications limit the reproducibility of pecan shell characterization studies (Gosselink, 2011).

In the present study, Nacono and Caddo crude extracts were extracted with acidified methanol (1% HCl v/v) to free, polymeric or bound form phenolics. The soluble components were analyzed by RP-HPPLC-DAD with the same method used to analyze their crude constituents. Acid hydrolysis removed interfering components in the chromatograms, resulting in the detection of two prominent and fully resolved peaks for all extracts (Figure 3.2).

Table 3.2. Major methanol soluble bioactive components elucidated by reverse phase HPLC in crude and acid hydrolyzed pecan shell extracts

Extract	Peak	Retention Time	Peak Area	% Area
HNA	1	4.9	6.3E+07	70.31
	2	6.3	1.3E+07	14.22
			Total	8.9E+07
HNE	1	4.9	5.2E+07	77.48
	2	6.3	1.0+07	15.33
			Total	6.7E+07
HCA	1	4.9	6.9E+07	67.03
	2	6.3	2.2E+07	21.86
			Total	1.0E+08
HCE	1	4.9	4.0E+07	71.17
	2	5.9	1.1E+07	19.34
			Total	5.7E+07
CNA	1	5.8	1.2E+08	30.31
	2	7.9	4.5E+08	11.42
	3	13.8	8.5E+06	2.15
			Total	3.2E+08
CNE	1	5.7	1.3E+08	46.79
	2	7.4	1.2E+08	44.95
	3	13.3	1.5E+07	5.37
			Total	2.6E+08

HNA = Acid hydrolyzed aqueous extracts from the Nacono cultivar
HNE = Acid hydrolyzed ethanol extracts from the Nacono cultivar
HCA = Acid hydrolyzed aqueous extracts from the Caddo cultivar
HCE = Acid hydrolyzed ethanol extracts from the Caddo cultivar
CNA = Crude aqueous extract from the Nacono cultivar
CNE = Crude ethanol extract from the Nacono cultivar

A peak at 4.9 min with a maximum absorption wavelength of 280nm was common in all extracts but was most abundant in aqueous extracts. This peak closely resembled gallic acid with Rt 5.0 min and max absorption at 272nm. The second major component eluted at Rt 6.3 with maximum absorption at 280 nm, which was not consistent with phenolic standards. It is hypothesized that this peak is a phenolic product derived from acidified methanol

extraction. The quantification of these peaks was not attempted, however retention times, and relative abundance is reported in Table 3.2 Rosa et al. 2011 identified only gallic and ellagic acid in acid-hydrolyzed acetonic extracts from pecan nutshell. In another study, Rosa et al. 2014 showed acetone: water (70:30 v/v) soluble epicatechin components in pecan shell are hydrolyzed to gallic and ellagic acid under acid conditions. HPLC data provided little analytical information to conclusively characterize shell bioactive components. Thus, more powerful analytical methods were employed.

3.3.4. Bioactive characterization by FIA-ESI-MS

Mass spectrometry is an analytical technique used to determine molecular masses of analytes by creating ions and separating them by their mass to charge ratio. This technique can provide structural information with molecular specificity unmatched by HPLC. There are only a few studies that have used mass spectrometry to characterize pecan shell bioactive components, despite its high analytical power. Rosa et al. 2011 determined gallic acid and ellagic acid to be the only phenolic compounds in acid hydrolyzed acetonic pecan shell extracts by RPHPLC-ESI-MS. Oligomeric proanthocyanadins were reported to exist in varying degrees of polymerization from 3 to 10 in shell extracts (Vazquez-flores et al., 2017).

In the present study, protonated and deprotonated ions produced using electrospray ionization with a typical fragmentation setting of acid hydrolyzed Nacono pecan shell extracts were monitored simultaneously with ion mode switching every second. Spectral data was digitally processed with Advion data express software. Background signal was subtracted from the peak ion chromatogram signal to improve the spectral resolution of the mass spectrums.

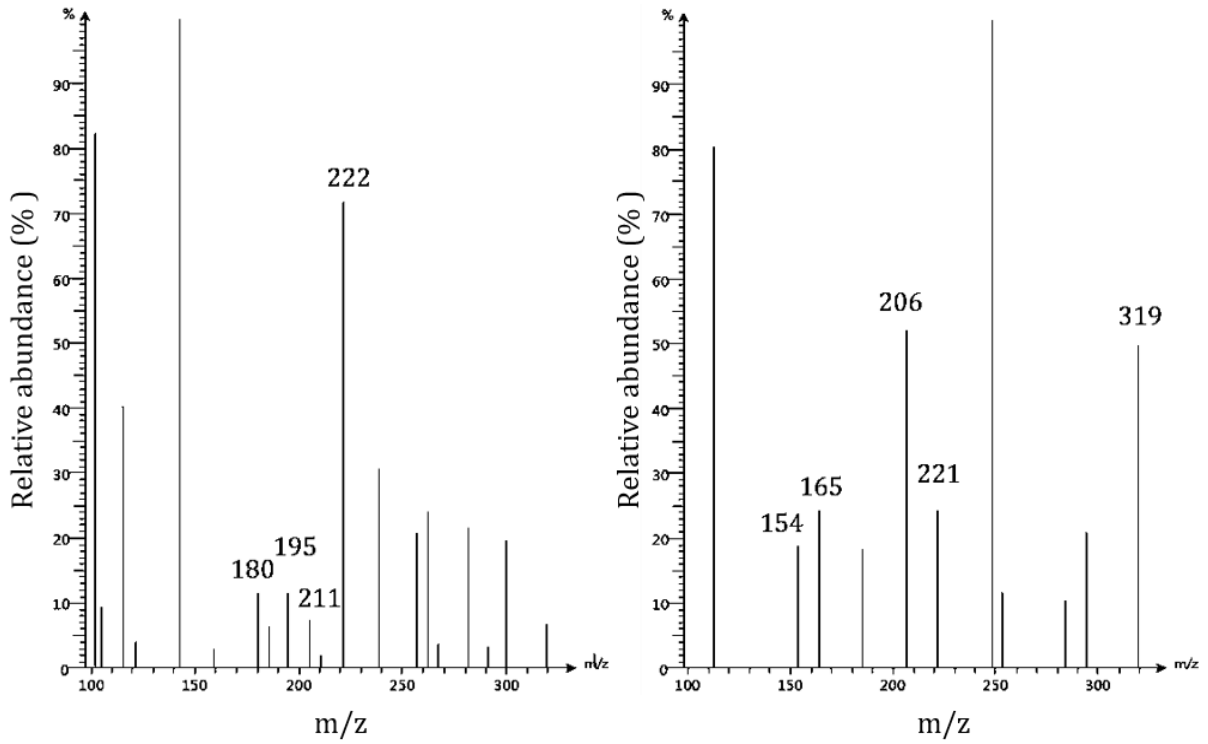

Figure 3.4. Mass spectrums by flow injection- electrospray ionization- mass spectrometry (FI-ESI-MS) of acid hydrolyzed (1% HCl v/v in methanol) ethanolic extracts of Nacono cultivar with ion mode switching every second. Protonated ions (Left): Coniferyl alcohol (179 u), phenolic 8-end G(β-O-4')G dilignol (m/z 195), Sinapyl alcohol S-lignol (210 u), and aliphatic 4-end of G(β-5')G dilignol (221 u). Deprotonated ions (Right): Vanillyl alcohol (154 u), guaiacylpropane (166 u), phenolic 8-end of G(β-β')G dilignol (m/z 206), aliphatic 4-end of G(β-5')G dilignol, and guaiacylglycerol-β-guaiacylether (320 u).

The major components identified were lignin degradation products lignols, dilignols, trilignols, and oligolignols and hydrolysis products from other polymeric components. Lignin is the second most abundant biomaterial on the planet and can be found in the secondary layer of plant cell walls. Lignin belongs to a large class of plant secondary metabolites called phenylpropanoids (Banoub et al., 2015). Structurally lignin is composed of repeating crosslinked units of lignols. Lignols are categorized according to the degree of oxygen substitution on the phenyl ring. The H-lignols (p-coumaryl alcohol) consists of one hydroxyl group. G-lignols (Coniferyl alcohol) contain one hydroxy and one methoxy group, and S-lignols (Sinapyl alcohol) display one hydroxyl and two methoxy groups Lignin is often

characterized by the ratio of H:G:S subunits (Doherty, Mousavioun, & Fellows, 2011; Heldt & Piechulla, 2011). Protonated ions of G(β-O-4')G fragments at m/z 195 (phenolic 8-end) and coniferyl alcohol g-structure lignol (aliphatic 4-end) at m/z 180, are likely products of lignin depolymerization by acidified methanol extraction. Deprotonated guiacylpropane (166 u) at m/z 165 was formed following the loss of formaldehyde (CH_2O, 30 u) from the later 8-phenolic end fragment (Haupert et al., 2012). Fragments of the aliphatic 4-end of G(β-5')G dilignol were detected in the protonated form at m/z 222 and in the deprotonated form at m/z 221.The least abundant fragment of G-structure dilignols observed was protonated phenolic 8-end of the β-β' resinol linkage at m/z 206 (Kiyota, Mazzafera, and Sawaya, 2012). Samples rich in different lignin monomer g-subunits, indicates a relatively high abundance of g-interunits present in pecan shell extracts (Banoub et al., 2015).

Other monomeric phenolics were identified. Deprotonated vanillyl alcohol (154 u) was detected at m/z 154 (Haupert et al., 2012). The most abundant deprotonated component was at m/z 143. Its molecular structure was not elucidated. The protonated form of sinapyl alcohol, the S-unit lignol, was detected in low abundance at m/z 211. Bonds associated with s-subunit dilignols are more resistant to cleavage. Low quantities of S-structure lignols may be due to low temperature and weak acid hydrolysis extraction conditions used (Banoub et al., 2015; Pandey & Kim, 2011). Protonated lignols were also detected at m/z 116, 143, and 160. Deprotonated ion at m/z 112 2-hydroxy-2,4-dienoate at m/z 112. The only identifiable dilignol was deprotonated guaiacylglycerol-B-guaiacylether dilignol at m/z 319 (Haupert et al., 2012; Kaiser & Benner et al., 2012). Proanthocyanidin A

Table 3.3. Identified bioactive components characterized by FIA-ESI-MS of acidified methanol (1% HCl) soluble components of pecan shell Nacono ethanol extracts.

m/z	Compound (MW)	peak area	% peak area	Maximum Intensity (c/s)
	ESI (-)			
112	2-Hydroxypenta-2,4-dienoate N.D.(113 u)	1.5E+06	3.9	1.2E+06
154	vanillyl alcohol (154 u)	3.4E+05	0.9	2.9E+05
165	guaiacylpropane (166 u)	3.5E+05	0.9	3.7E+05
206	phenolic 8-end of G(β-β')G dilignol	3.6E+05	0.9	8.0E+05
221	aliphatic 4-end of G(β-5')G dilignol	4.2E+05	1.1	3.7E+05
248	n.d.	1.6E+06	4.1	1.5E+06
319	guaiacylglycerol- β -guaiacylether (320 u)	8.9E+05	2.3	7.6E+05
	ESI (+)			
116	n.d.	1.2E+07	6.3	8.4E+06
143	n.d.	2.7E+07	14.7	2.1E+07
160	n.d.	8.1E+05	0.4	6.1E+05
180	coniferyl alcohol G-lignol (180 u)	3.7E+06	2.0	2.4E+06
195	phenolic 8-end G(β-O-4')G dilignol	5.2E+06	2.8	2.4E+06
211	sinapyl alcohol S-lignol (210 u)	7.8E+05	0.5	3.8E+05
222	aliphatic 4-end of G(β-5')G dilignol	1.9E+07	10.5	1.5E+07
593	proanthocyanadin A (593 u)	1.0E+06	0.5	5.1E+05

*n.d.=not determined

was detected in low abundance (peak area 0.5 %) in the positive ion mode at m/z 593. Mass spectrums of deprotonated ions between 300-1200 m/z showed evidence of highly polymerized components. There was a low abundance of components greater than 500 u detected in the positive ion mode. Various phenylpropanoid derivatives were the main components in ethanolic pecan shell extracts.

3.4. Conclusions

Among 20 tested cultivars, shell extracts from Caddo provided highest levels of phenolics and antioxidant activity (Folin-Ciocalteu and DPPH). Extracts obtained by solid-liquid extraction with ethanol were significantly higher in phenolics, compared to those obtained using distilled water; however, no significant difference was observed in antioxidant activity. The major components in ethanolic extracts identified by FIA-ESI-MS

were a range of phenylpropanoid derivatives including phenolic acids, flavonoids, and lignols with varying degrees of polymerization.

3.5. References

1. Banoub, J., Delmas, G. Jr., Joly, N., Mackenzie, G., Cachet, N., Benjelloun-Mlayah, B., & Delmas, M. (2015). A critique on the structural analysis of lignins and application of novel tandem mass spectrometric strategies to determine lignin sequencing. *J. Mass Spectrom.*, *50*, 5-48. doi: 10.1002/jms.3541

2. Brand-Williams, W., Cuvelier, M.E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology 28* (1), 25-30. doi: 10.1016/S0023-6438(95)80008-5

3. Chun, K. O., & Kim, D. (2004). Consideration on equivalent chemicals in total phenolic assay of chlorogenic acid-rich plums. *Food Research International*, *37*, 337-342. doi: 10.1016/j.foodres.2004.02.001

4. Doherty, W. O. S., Mousavioun, P., & Fellows, C. M. (2011). Value-adding to cellulosic ethanol: lignin polymers. *Ind. Crop Prod.*, *33*(2), 259-276. doi: 10.1016/j.indcrop.2010.10.022

5. Gosselink, R. J. A. (2011). Lignin as a renewable aromatic resource for the chemical industry. PhD Thesis, Wageningen University, Wageningen, NL.

6. Hagerman, A. E. (2002). Tannin Chemistry. Oxford OH: Department of Chemistry and Biochemistry, Miami, University.

7. Haupert, L. J., Owen, B. C., Marcum, C. L., Jarrell, T. M. , Pulliam, C. J., Amundson, L. M., Narra, P., Aqueel, M. S. , Parsell, T. H., Abu-Omar, M. M., & Kenttamaa, H. I. (2012). Characterization of model compounds of processed lignin and the lignome by using atmospheric pressure ionization tandem mass spectrometry. *Fuel*, *95*, 634-641. doi: 10.1016/j.fuel.2011.12.015

8. Heldt, H. and Piechulla, B. (2011). Phenylpropanoids comprise a multitude of plant secondary metabolites and cell wall components**.** 431-449. 10.1016/B978-0-12-384986-1.00018-1

9. Kaiser, K. & Benner, R. (2012). Characterization of Lignin by Gas Chromatography and Mass Spectrometry Using a Simplified CuO Oxidation Method. *Anal. Chem., 84*, 459-464. doi: 10.1021/ac202004r

10. Kiyota, E., Mazzafera, P., Sawaya, A. C.(2012). Analysis of soluble lignin in sugarcane by ultrahigh performance liquid chromatography tandem mass spectrometry with a do-it-yourself oligomer database. *Anal. Chem*., *84*(16), 7015-7020. doi:10.1021/ac301112y

11. Lerma-Herrera, M. A., Núñez-Gastélum, J. A., Ascacio-Valdés, J., Aguilar, C. N., Rodrigo-García, J., Díaz-Sánchez, A. G., … de la Rosa, L. A. (2017). Estimation of the Mean Degree of Polymerization of Condensed Tannins from the Kernel and Shell of Carya illinoinensis by HPLC/MS and Spectrophotometric Methods. *Food Analytical Methods, 10*(9), 3023–3031. doi:10.1007/s12161-017-0866-6

12. Malik, N., Perez, J.L., Lombardini, L., Cornacchia, R., Cisneros-Zevallos, & Braford, J. (2009). Phenolic compounds and fatty acid composition of organic and conventional grown pecan kernels. *J Sci Food Agric, 89*, 2207-2213. doi:10.1002/jsfa.3708

13. Pandey, M. P., & Kim, C. S. (2010). Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. *Chemical Engineering Technology*, *34* (1), 29-41. doi: 10.1002/ceat.201000270

14. Prado, A. C. P., Aragão, A. M., Fett, R., & Block, J.M. (2009). Antioxidant Properties of Pecan Nut [Carya illinoinensis (Wangenh.) C. Koch] Shell Infusion. *Grasas y Aceites*, *60* (4), 330-335. doi: 10.3989/gya.107708

15. Prado, A. C. P., Silva, Manion, B. A., Seetharaman, K., Deschamps, F. C., Arellano, D. B., & Block J. M. (2013). Relationship between antioxdiant properties and chemical composition of the oil and the shell of pecan nuts [*Carya illinoinensis* (Wangenh) C. Koch]. *Industrial Crops and Products*, *45*, 64-73. doi: 10.1016/j.indcrop.2012.11.042

16. Prado, A. C. P., Silva, H.S., Silveira, S.M., Barreto, P. L. M., Vieira C. R. W., Maraschini, M., Ferreira, A. R. S., & Block J. M. (2014). "Effect of the extraction process on the phenolic compounds profile and the antioxidant and antimicrobial activity of extracts of pecan nut [*Carya illinoinensis* (Wangenh) C. Koch] shell." *Industrial Crops and Products, 52*, 552-561. doi: 10.1016/j.indcrop.2013.11.031

17. Rosa, L. A., Alvarez-Parrilla, E., & Shahidi, F. (2011). Phenolic Compounds and Antioxidant Activity of Kernels and Shells of Mexican Pecan (*Carya illinoinensis*). *J. Agric. Food Chem.*, *59*, 152-162. doi: 10.1021/jf1034306

18. Rosa, L. A., Vazquez-Flores, A. A., Alvarez-Parrilla, E., Rodrigo-Garcia, J., Medina-Camposc, O. N., A´ vila-Nava, A., Gonza´ lez-Reyes, S., & Pedraza-Chaverri, J. (2014). Content of major classes of polyphenolic compounds, antioxidant, antiproliferative, and cell protective activity of pecan crude extracts and their fractions. *Journal of*

Functional Foods, *7*, 219-228. doi: 10.1016/j.jff.2014.02.008

19. Sánchez-Rangel, J. C., Benavides, J., Heredia, J. B., Cisneros-Zevallos, L., & Jacobo-Velázquez, D. A. (2013). The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. *Analytical Methods*, *5*, 5990-5999. doi:10.1039/C3AY41125G

20. Singleton, V. L., Orthofer, R. & Lamuela-Raventos, R. M. (1999) Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. *Methods in Enzymology, 299*, 152-178. Doi: 10.1016/s0076-6879(99)99017-1

21. Soobrattee, M. A., Neergheen, V.S., Luximon-Ramma, A., Aruoma, O. I., & Bahorun, T. (2005). Phenolics as potential therapeutic agents: Mechanism and actions. *Fundamental and Molecular Mechanisms of Mutagenesis*, *579*, 200-213. doi: 10.1016/j.mrfmmm.2005.03.023

22. Statista. (2019). Worldwide sales of organic foods 1999-2017 (in billion U.S. dollars). Retrieved from https://www.statista.com/statistics/273090/worldwide-sales-of-organic-foods-since-1999/

23. United States Department of Agriculture Economic Research Service (ERS). (2017). Organic Market Overview. Retrieved from https://www.ers.usda.gov/topics/natural-resources-environment/organic-agriculture/organic-market-overview.aspx

24. United States Department of Agriculture National Agriculture Statistics Service (NASS). (2018). National Statistics for Pecan. Retrieved from https://www.nass.usda.gov/Statistics_by_Subject/result.php?7345CB67-26CC3917AF5BC7828216D668&sector=CROPS&group=FRUIT%20%26%20TREE%20NUTS&comm=PECANS

25. Vazquez-Flores, A. A., Wong-Paz, J. E., Lerma-Herrera, M.A., Martinez-Gonzalez, A. I., Olivas-Aguirre, F. J., Aguilar, C. N., Wall-Medrano A., Gonzalez-Aguilar, G. A., Alvarez-Parrilla, E., & Rosa, L. A. (2017). Proanthocyanidins from the kernel and shell of pecan (Carya illinoinensis): Average degree of polymerization and effects on carbohydrate, lipid, and peptide hydrolysis in a simulated human digestive system. *J. Functional Food*, *28*, 227-234. doi: 10.1016/j.jff.2016.11.003

26. Villarreal-Lozoya, J. E., Lombardini, L., Cisneros-Zevallos, & L. (2007). Phytochemical constituents and antioxidant capacity of different pecan [*Carya illinoinensis* (Wangenh.) K. Koch] cultivars. *Food Chem., 102*, 1241-1249. doi:

10.1016/j.foodchem.2006.07.024

27. Worley, R.E. (1994). Pecan Physiology and Composition. In: Santerre C.R. (eds) *Pecan Technology* (pp. 39-48). Springer, Dordrecht.

4. CONCLUSION

The results from this study indicate that pecan shell by-products have potential to be used as a natural source of antioxidant in various food applications. Bioactive components obtained by solid-liquid extraction with distilled water for 30 min at 98^{o}C, and ethanol for 1 hr at 22^{o}C with constant mixing at 160 rpm of 20 pecan cultivars grown in Louisiana had high phenolic content and antioxidant activity measured by the Folin-Ciocalteu and DPPH free radical assays, respectively. Cultivar significantly affected (P<0.05) total phenolic content and antioxidant activity. Extraction method significantly affected (P<0.05) phenolic content, but not antioxidant activity. Total phenolic content of shell ethanolic extracts ranged from 304.2 (*Caddo*) to 153.54 (*Cherokee*) mg GAEg^{1} dry extract and were significantly greater (P<0.05) than those obtained by aqueous extraction, which ranged from 253.75 (*Curtis*) to 114.63 (*Jackson*). The antioxidant activity of ethanolic extracts ranged from 840.6 (*Maramec*) to 526.74 (*Caper Fear*) mg TEg^{-1}, while aqueous extracts ranged from 934.9 (*Curtis*) to 468.3 (*Elliot*) mg TEg^{-1} dry extract.

Characterization of crude aqueous and ethanolic extracts of the Caddo and Nacono pecan cultivars was not achieved by reverse phase high performance liquid chromatography (RP-HPLC). Chromatograms of crude extracts resulted in a single broad-shouldered peak. This was mostly attributed to interfering materials attributed to lignocellulosic and other glycoside bound components. Crude extracts were extracted with acidified methanol (1% HCL), which resulted in the removal of the interfering material and allowed for the elution of two components in either extract. The first and most abundant peak was attributed to gallic acid, while the other peak did not resemble phenolic standards.

Acid hydrolyzed Nacono ethanolic extracts were further analyzed by flow injection electrospray ionization mass spectrometry with detection in the positive and negative ion modes. The major components identified between 100-1200 m/z were lignin degradation products with varying degrees of polymerization. Monolignols corresponding to fragmentation of g-structure dilignols were numerous. The antioxidant activity of pecan shell extracts is attributed to a wide variety of bioactive compounds from the class of phenylpropanoids. The significance of these finding is the potential to create new revenue streams for shell by-product, thereby increasing the economic value of the Louisiana pecan crop.

SUPPLEMENTARY DATA

Table S1. Extract yield of pecan shell extracts from 20 cultivars obtained by distilled water and ethanol extraction procedures.

Cultivar	Extract yield mg dry extract/g defatted shell powder	
	Aqueous extracts	Ethanolic extracts
Desirable	263	25
Caddo	492	281
Elliot	286	175
Nacono	214	305
Oconee	307	176
Pawnee	260	240
Point Coupee	481	144
Curtis	257	66
Kiowa	165	174
Moreland	203	162
Cherokee	351	314
Schley	201	63
Success	145	35
Sumner	222	3
Gloria Grande	221	139
Cape Fear	394	372
Creek	96	273
Maramec	253	50
Jackson	89	265
Melrose	90	65
Average	250	166

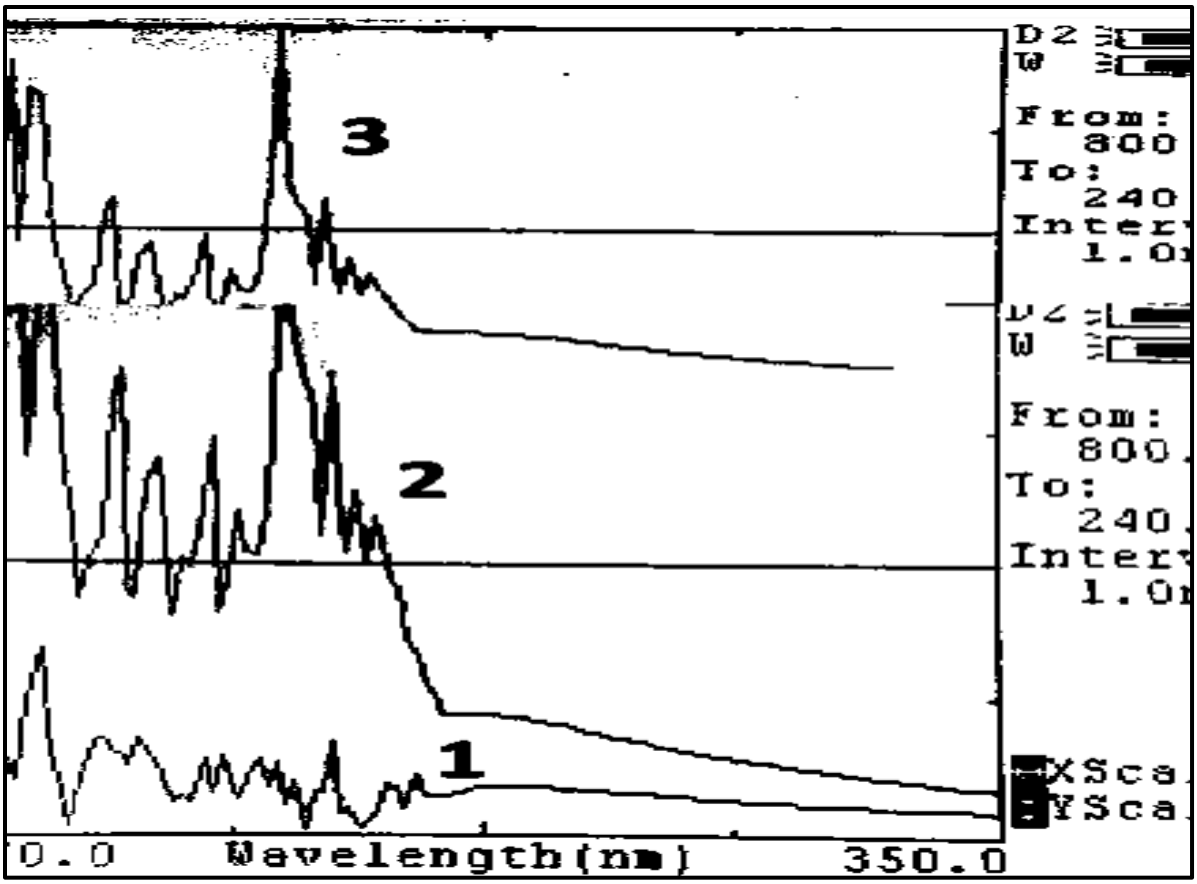

Figure S1. Absorbance spectrum (240 – 350 nm) of Caddo ethanolic extract fractions 1-3 separated by lipophilic LH-20 Sephadex resin.

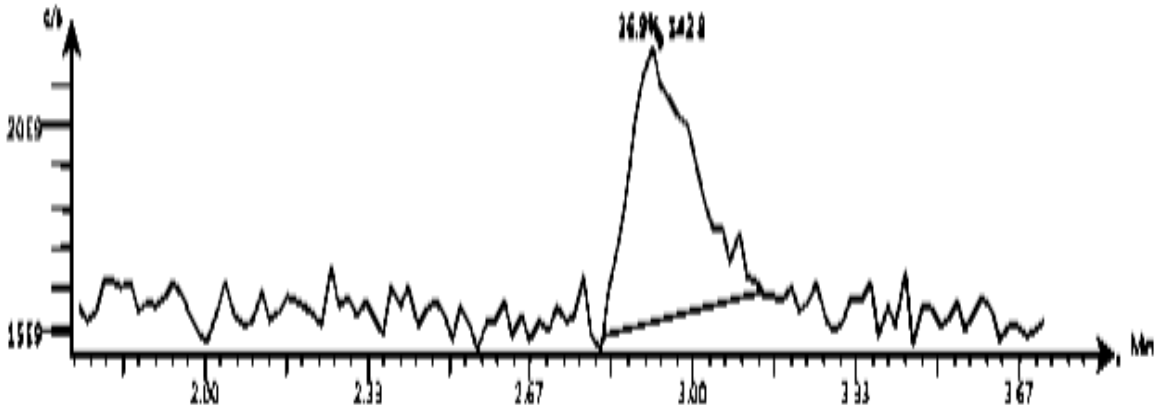

Figure S2. Total ion chromatogram of positive and negative ions produced using electrospray ionization of Nacono ethanolic extract using a normal fragmentation setting.

VITA

Cameron Cason was born in Baton Rouge, Louisiana. He received his Bachelor's in Food Science Technology from Louisiana State University in 2017. During his time as an undergraduate he conducted research to identify foodborne pathogens present on harvested pecans. He is currently pursuing his Master's in Food Science and Technology at Louisiana State University. He is a graduate research assistant in the School of Nutrition and Food Science. He plans to graduate in May of 2019.